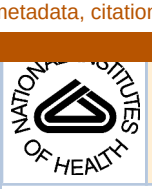

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Aqueous Humor Outflow:

What Do We Know? Where Will It Lead Us?

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The second annual ARVO/Pfizer Ophthalmic Research Institute conference was held Friday and Saturday, April 28 and 29, 2006, at the Fort Lauderdale Grande Hotel and Yacht Club, Fort Lauderdale, Florida. The conference, funded by The ARVO Foundation for Eye Research through a grant from Pfizer Ophthalmics, provided an opportunity to gather experts from within and outside ophthalmology to develop strategies to improve research and clinical care in areas of ophthalmology related to preventable vision loss and blindness. This year's conference focused on aqueous humor outflow.

A working group of 31 researchers on aqueous outflow, 8 scientists working on interests other than aqueous humor outflow but with expertise in areas relevant to the field, and 11 observers from ARVO, Pfizer, and clinical and basic ophthalmic research convened on April 28, 2006, to evaluate the current understanding of the aqueous humor outflow pathway. The goals were to compare similarities between ocular aqueous humor outflow and fluid flow in other tissues of the body, to critique conventional ideas, to identify the most important scientific questions, and to discuss strategies to answer these questions.

The meeting format emphasized discussion and concentrated on questions within four general areas of outflow research:

Session I: Conventional Outflow: Cell Function in the Aqueous Humor Outflow Pathway

Session II: Conventional Outflow: Role of the Extracellular Matrix

Session III: Uveoscleral Outflow: The Other Pathway

Session IV: Normal Versus Primary Open Angle Glaucoma (POAG): What Is the Cause of Elevated Pressure?

Each session began with a 10-minute overview by an outflow researcher followed by a 30-minute talk by one of the outside experts. Parallels between their fields of expertise and the eye were included in these talks. Invited outside experts covered several areas of research, including vascular endothelial cell biology (Peter Davies, PhD, University of Pennsylvania, Philadelphia), matricellular proteins (Paul Bornstein, MD, University of Washington, Seattle), renal and lymphatic biology (Donatscho Kerjaschki, MD, University of Vienna, Austria, and David Zawiega, PhD, Texas A & M University, College Station, Texas), oxidative damage mechanisms (James Mitchell, PhD, National Institutes of Health, Bethesda, Maryland), advanced glycation end-products and aging (Alan Stitt, PhD, Queens University, Belfast, Northern Ireland), cell adhesion (Benjamin Geiger, PhD, Weizmann Institute of Science,

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Rehovot, Israel), and cellular tight junctions (Eveline Schneeberger, MD, Massachusetts General Hospital, Boston, Massachusetts).

The remainder of each session was used to discuss questions pertinent to the main topic and provided an opportunity for attendees to voice their opinions and work together to define questions that are still unanswered. The lively discussions were successful in defining old and new ideas that are essential to a better understanding of aqueous humor outflow. This conference summary highlights the ideas discussed and introduces areas that need further exploration.

Aqueous Humor Outflow

Aqueous humor is produced by the ciliary body epithelium in the posterior chamber and flows into the anterior chamber. It is a complex mixture of electrolytes, organic solutes, growth factors, and other proteins that supply nutrients to the nonvascularized tissues of the anterior chamber (i.e., trabecular meshwork [TM], lens, and corneal endothelium). The aqueous exits the eye either through the TM into Schlemm's canal and aqueous veins (conventional pathway) or through the ciliary muscle and other downstream tissues (unconventional pathway).

The conventional pathway consists of the TM and Schlemm's canal. The TM is a filter made up of extracellular matrix (e.g., collagens), most of which is organized into a network of beams covered by endothelial-like trabecular cells. As aqueous humor moves from the interior part of the meshwork (uveal) to the middle (corneoscleral) and to the exterior side (juxtacanalicular) adjacent to the inner wall of Schlemm's canal, the porosity of the tissue decreases. It is thought that the resistance to aqueous outflow occurs at the junction where the TM meets the inner wall of Schlemm's canal.[1] If this is the site of outflow resistance, what role do the cells and the extracellular matrix have in maintaining the correct pressure in the eye?

Conventional Outflow: Cell Function in the Aqueous Humor Outflow Pathway

The session centered around three questions: What is the evidence that the pathophysiology of ocular hypertension is cell mediated, how do outflow cells deal with stress, and how does the aqueous humor enter Schlemm's canal? The discussion revealed several areas in which research could aid in our current understanding of the outflow pathway cells' role in aqueous humor outflow.

Basic to the understanding of how the conventional pathway works is knowing whether there are different cell populations in the TM and Schlemm's canal, what these cells produce, and how they function. Although advances have been made toward understanding the biochemical and molecular characteristics of the trabecular cells, still needed is the identification of molecules unique to specific cell phenotypes. Schlemm's canal cells express CD31, typical of vascular endothelial cells.[2] In contrast, some but not all trabecular cells express chitinase 3-like 1 (CH3L1),[3] suggesting cellular heterogeneity in trabecular cells. Identification of additional molecules unique to Schlemm's canal cells and TM cells would enable the use of more sophisticated techniques to target tissue-specific knockin or knockout gene expression or silencing of tissue-specific genes in perfusion organ culture of anterior segments or in animal models. The use of laser capture microdissection[4] followed by microarray analysis may be a valuable tool for dissection of individual cell types and as an aid in the identification of molecular markers specific for each cell type.

Identifying the means by which the TM cells sense and react to their environment (fluid-flow, shear stress, or stretch) will improve our understanding of how aqueous outflow is regulated. Do all TM cells react in a similar fashion to environmental stimuli or stress? Cell-environment interactions may include stimuli at both the apical and basal sides of the cell. Changes that

affect the apical side of a cell may alter cellular responses at the basal side, resulting in a decentralized signaling pattern.[5] In vascular endothelial cells, the main apical stimulus is due to shear stress as blood flows over the apical surfaces. Vascular endothelial cells respond by aligning with the local flow direction, whereas areas of "disturbed" flow (without a preferred flow direction) do not have aligned cells.[6] Schlemm's canal cells are subject to shear stresses of a magnitude similar to vascular endothelial cells and can also become arranged in an aligned pattern near collector channels.[7] It is interesting that Schlemm's canal cells are exposed to a basal-to-apical pressure difference, opposite the situation of vascular endothelial cells. Does gene expression change in Schlemm's canal cells when exposed to shear stress? Are these changes similar to those of vascular endothelial cells during shear stress? How do aligned Schlemm's canal cells differ from Schlemm's canal cells that are not aligned?

How does the aqueous humor move from the TM into Schlemm's canal? Several structures have been identified that appear to be associated with fluid movement within the conventional pathway. Pores have been observed within and between Schlemm's canal cells.[8-11] Pores are more numerous in eyes with higher outflow facility, suggesting a role in aqueous fluid outflow.[10] However, pores increase with fixation, suggesting that some may be artifacts.[8,9,11] The other structure associated with changes in fluid movement in the canal are giant vacuoles. These are pressure-sensitive cellular protrusions that occur within and between canal cells. Similar structures are found in arachnoid villi, and probably arise because of the "backward," or basal-to-apical, fluid movement in these regions. Whether giant vacuoles serve as conduits for aqueous entry into the canal in conjunction with pores or instead function as a mechanism to sense pressure by stretching and allow greater fluid flow in the neighboring intercellular junctions is unknown. New techniques are needed to help identify whether these structures are physiologic. Two-photon scanning laser confocal microscopy[12,13] may be useful for visualizing the cells in the inner wall of Schlemm's canal in real time. The use of fluorescence recovery after photobleaching may be useful in tracking fluorescent tracers distributed throughout the perfusing fluid.[14-16] If giant vacuoles are determined to be normal physiologic structures, what cellular dynamics would lead to giant vacuole formation? What is the function of the cytoskeleton and the cell membrane, and how do the cells attach and detach from the extracellular matrix?

An additional conceptual question that arose during discussion of the cells in Schlemm's canal was why the lining of the canal contains tight junctions if it must be leaky enough to allow bulk flow of aqueous from the TM into the canal? Although tight junctions in all tissues allow some water to move through them, the aqueous outflow system is quite "leaky" in comparison to other vascular endothelia (e.g., hydraulic conductivity of a lung capillary is approximately 3×10^{-11} $cm^2 \cdot s/g$, whereas the hydraulic conductivity of the TM is approximately 7000×10^{-11} $cm^2 \cdot s/g$).[17] Determination of the composition of tight junctions (e.g., claudins, occludins),[18] gap junctions, and cell adhesion molecules is necessary to clarify the cellular structure and function within the TM and Schlemm's canal.

Conventional Outflow: Role of the Extracellular Matrix

Although cells produce and regulate the amount of extracellular matrix, the matrix works with cells to help regulate cell function. This process occurs through interactions with integrins and cell adhesion molecules.[19] Discussion focused on (1) evidence supporting the involvement of extracellular matrix in aqueous humor outflow, (2) which extracellular matrix molecules could control outflow resistance, and (3) whether POAG is caused by a loss of trabecular cell-extracellular matrix interaction.

The extracellular matrix of the TM is made up of an intricate arrangement of collagen, laminin, fibronectin, proteoglycans, glycosaminoglycans (GAGs), and matricellular proteins. Matricellular proteins (e.g., thrombospondins, secreted protein acidic and rich in cysteine

[SPARC], tenascin) are nonstructural adaptor proteins that modulate cell-matrix interactions.[20] Matricellular proteins are generally found during development and after tissue repair. In the normal TM, these proteins are found in relatively high abundance, but their specific roles within the tissue is unclear.[21,22] Why does the meshwork contain these adaptor proteins? Are they present in the TM because of stresses—particularly the stretching and configuration changes seen with variations in intraocular pressure (IOP)? Does this indicate a reaction to potential damage caused by this tissue movement? Use of antisense or small interfering (si)RNAs to decrease the amount of specific molecules may help to improve our understanding of matricellular protein function in the TM. For example, a reduction in SPARC could lead to a reduction in the deposition of extracellular matrix molecules.[22] Insight into the function of these matricellular proteins within the TM may help to further our understanding of cell-extracellular matrix function within this tissue.

The role of GAGs in outflow resistance remains unclear. GAGs are negatively charged molecules found primarily on the surfaces of cells and in the extracellular matrix. Molecules in this family can create increased viscosity within solutions due to their extended conformation. In cartilage, one role of GAGs is to hold water and slowly release it under pressure.[23] Early studies of bovine eyes suggested that hyaluronic acid may serve a similar function of controlling fluid motion in the eye, since perfusion with hyaluronidase decreases outflow resistance.[24] However, more recent studies have produced mixed findings.[25,26] Differences in enzyme purity may explain the disparate findings, because enzyme preparations may be contaminated with other proteases or may be less specific than thought and hence digest more than the GAGs of interest.

Evidence supporting the role of GAGs or proteoglycans in outflow resistance has been found in eyes with POAG. These eyes have less hyaluronic acid and more chondroitin sulfate than do healthy eyes.[27] Fibronectin undergoes changes in splice variants in trabecular cells undergoing stretch, losing some of the GAG-binding sites, suggesting that this may be part of a self-regulating feedback mechanism to decrease outflow resistance if the meshwork is deformed by high IOP (Gregory K et al. *IOVS* 2006;47:ARVO E-Abstract 1341). Does the TM contain substantial amounts of GAGs making these molecules responsible for outflow resistance? At present, histologic techniques to verify the amount of GAGs in the TM may cause a partial or complete loss of these molecules during processing. Preservation of the tissue by freezing with isopentane may provide a deeper and more instant freeze and help in the preservation of these molecules. Also, the use of newer microscopy techniques, such as environmental scanning electron microscopy,[28] may provide better preservation of the extracellular matrix, enabling visualization of the GAG molecules.

The TM is anchored by ciliary muscle tendons and fine elastin fibers that connect to the endothelium of Schlemm's canal. Contraction, tension, or relaxation of the ciliary muscle influences the shape of the TM, ultimately modifying aqueous humor flow through the meshwork and Schlemm's canal.[29-31] This hydraulic-like system may influence cellular responses—particularly those of the actin cytoskeleton. The use of cytoskeleton-modifying agents (H7 and latrunculin A and B) has been shown to lower IOP, suggesting the actin cytoskeleton as a target for regulating aqueous outflow.[32-35] The TM itself has contractile ability that works antagonistically toward the ciliary muscle's effect on outflow.[36] Isolated strips of bovine TM contract in the presence of carbachol or endothelin and decrease outflow facility.[37]

Are the cells of the conventional pathway (trabecular or Schlemm's canal) or the extracellular matrix around the cells responsible for conventional outflow resistance? This question has been debated for more than 100 years. Discussion of the data supporting both sides of this debate have suggested that it is not an "either/or" question. Evidence now suggests that both cells and

the extracellular matrix affect outflow resistance.[38,39] Because the cells are responsible for creating the extracellular matrix, they can be considered the ultimate regulator of IOP. However, on a longer time scale (hours or more), the extracellular matrix seems likely to modulate outflow resistance significantly. Therefore, both the cells and the extracellular matrix are essential in the task of maintaining aqueous outflow.

Uveoscleral Outflow: The Other Pathway

Since its first description in the 1960s, the unconventional pathway (uveoscleral outflow) has been more difficult to study than the conventional pathway and hence is less well understood. The conference discussion topics focused on what precisely is the uveoscleral outflow pathway, how are relevant physiological parameters within uveoscleral outflow measured (e.g., flow rate), how much does it contribute to total outflow, and can we use the uveoscleral pathway better?

In uveoscleral outflow, aqueous humor enters the ciliary muscle and exits through the supraciliary space and across the anterior or posterior sclera, through the emissarial canals around the vortex veins, or into the choroidal vessels.[40] The relative proportion of aqueous humor entering each site is controversial. Until recently, the uveoscleral outflow pathway was largely considered a passive and minor route for aqueous humor outflow. It is now known that uveoscleral drainage can account for up to 60% of the total aqueous humor drainage in nonhuman primates.[41] In other animal species, the amount varies from 3% to 8% in rabbits[40] and up to 80% in mice.[42] In humans, reports range from 4% to 60%.[43,44] Because age tends to decrease uveoscleral flow in humans,[45] the conventional outflow pathway must compensate for the effect of aging, to prevent an increase in IOP. Identification of the changes that occur in the uveoscleral pathway with age is needed to explain the reduced uveoscleral flow.[46] Currently, there are no direct, noninvasive methods for determining uveoscleral outflow. Calculations based on the modified Goldmann equation are the only way to estimate uveoscleral outflow in clinical studies. New techniques for the measurement of uveoscleral flow are clearly needed.

Using the uveoscleral pathway for therapeutic purposes could involve both lowering of IOP and drug delivery via a transscleral approach. Current understanding of the mechanism of the IOP decrease caused by prostaglandin treatments involves permeability changes in the ciliary body.[44] Could new drugs change the permeability even more, resulting in lower IOP? Can scleral permeability be altered by drugs such as prostaglandins to allow diffusion of drugs from the orbit into the eye?

Normal versus POAG: What Is the Cause of Elevated Pressure?

POAG is a heterogeneous disease that becomes increasingly common with age. Many factors probably contribute to the disease, ranging from loss of “normal” trabecular cells to abnormal trabecular cell function. With the increasing sophistication of techniques in molecular biology and biochemistry, analysis of TM specimens and aqueous humor proteins has revealed a variety of molecules that differ between normal and glaucomatous eyes. A recent proteomics study found 368 proteins in the TM, with 52 present only in glaucomatous TM and 177 present only in normal TM.[47] Several molecules (TGFβ2, VEGF, endothelin, PAI, and soluble CD44) are elevated in the aqueous humor of POAG when compared to normal aqueous humor.[48-52] These molecules may influence trabecular cells to change their “usual” phenotype. For example, trabecular cells could respond to elevated aqueous humor levels of TGF-β2 by secreting additional extracellular matrix molecules.[53] Although the cellular response to TGF-β2 would be normal, the ultimate outcome may be an abnormal accumulation of extracellular matrix within the TM. However, caution must be applied in the interpretation of the “unique” or differentially expressed molecules in the TM and aqueous humor in POAG, because the

effects of glaucoma medications on the molecular composition of the TM and aqueous humor are not fully understood. Furthermore, in investigations of specific protein levels in aqueous humor, the relative concentrations of the molecules should be expressed as a proportion of total aqueous protein, to help gauge whether the aqueous humor is mixed with contaminating proteins.

As the population ages, more attention is being paid to damage caused by various forms of oxidative stress. Studies of cardiovascular diseases, cancer, diabetes, Alzheimer's disease, and eye diseases such as cataract and advanced macular degeneration have identified oxidative stress as a potential factor involved in the progression of the disease.[54-56] In POAG, it has been reported that patients have decreased total plasma antioxidant capacity (TRAP) and increased superoxide dismutase (SOD) levels, suggesting an increase in oxidative stress.[54] Oxidative DNA damage has been reported in the TM of eyes of patients with glaucoma.[57] Endothelial leukocyte adhesion molecule (ELAM-1), produced after cells release NF-κB in response to oxidative stress, has been identified as a glaucoma marker in trabecular cells, suggesting that cells within the outflow pathway are under oxidative stress.[58] What is the cause of the oxidative damage and how does this alter the outflow pathway? Has the antioxidative potential of aqueous humor been compromised, enabling an imbalance between free radical production and antioxidative defense mechanisms? Improved understanding of the oxidative stresses, the antioxidant potential, and the effect of oxidative damage on the cellular function of the outflow pathway will help identify the possible role of oxidative stress in the etiology of POAG.

Oxidative stress can also occur through the accumulation of advanced glycation end (AGE) products.[59] Chemical modifications to carbohydrates (reducing sugars) and free amino groups of proteins may produce nonenzymatic protein cross-links. Accumulation of AGE products may interfere with the structural properties of the protein, potentially resulting in deleterious effects. AGE products have been found in the cornea, lens, vitreous, and retina, and an increase in AGE products in age-related macular degeneration suggests that they have a role in many ocular diseases.[59] In the meshwork, tissue transglutaminase is present and can be induced in cultured trabecular cells by TGFβ1 and -β2 treatment,[60] which causes polymerization of fibronectin and may result in irreversibly cross-linked extracellular matrix proteins. The presence of AGE products in the outflow pathways and role of AGE in glaucoma is unknown. It is interesting to note that accelerated AGE production occurs in diabetes.[61-63] Whether glaucoma is more common in individuals with diabetes is still controversial. Older studies found a relationship,[64,65] whereas the recent Ocular Hypertension Treatment Study did not.[66]

Additional Discussion Topics

Dynamics of the Outflow System

The outflow pathways maintain a stable IOP despite transient elevations of IOP that occur due to various eye movements (blinking, squeezing the eyes shut, rubbing the eyes).[67] How does the TM prevent too much aqueous outflow during these short periods of high pressure, yet allow a constant, low level of aqueous outflow? How does the TM handle levels of stress (stretch, shear)? Is the system of the ciliary muscle, TM, and Schlemm's canal responsible for handling these transient changes, or can the cells of the conventional pathway respond rapidly enough to produce the changes? What happens to the actin cytoskeleton during these events? Does outflow resistance change at night? Recent studies finding elevation of IOP at night[68] are surprising, in view of the decrease in aqueous humor flow.[69]

Analysis of Current Models

Given our growing understanding of the importance of cell-extracellular matrix interactions on cellular physiology, cell culture conditions that do not match the normal cell-extracellular matrix profile may be outdated.[70] Can cell cultures become more physiologic? Comparisons of cultured cells in different conditions are needed, with the ultimate goal of matching the characteristics (cell markers) of fresh, noncultured cells. Comparisons should include medium (aqueous humor versus fetal bovine serum), substrate (extracellular matrix versus plastic), and oxygen (aqueous 5% versus room air 21%).[71,72]

Animal Models

Although POAG is mainly a human disease, naturally occurring glaucoma has been identified in rhesus monkeys[73] and certain strains of dogs.[74] Development of animal models that mimic the disease would advance outflow research. A systematic screening of the eyes in existing knockout mouse models may find important glaucoma-associated changes or anatomic changes in the outflow pathways that will help to improve our understanding of outflow physiology. Transgenic and conditional knockout strategies (CRE/LOX)[75] may be useful in regulating gene expression in a tissue-specific manner and may enable analysis of extracellular matrix genes and their effect(s) on the outflow pathways.

An alternative approach would be to use chemical mutagenesis[76] and identify abnormal outflow in mice. This method would enable the screening of many point mutations within the mouse. The identification of a glaucoma phenotype in the mouse would provide the foundation for a genetic survey and identification of genes associated with the disease.

Summary

Many questions remain in the study of the normal aqueous outflow pathway and understanding of what is altered in POAG. One of the goals of the conference was to identify future areas of study that will improve our understanding of aqueous humor outflow. These questions have been summarized in Table 1.

Finally, suggestions were made that the role of ARVO in research could be expanded in developing a consensus on terminology and techniques. “Giant vacuoles” of Schlemm’s canal endothelium are not truly intracytoplasmic vacuoles, and the term and concept was initially confusing to the outside scientists at the conference. An ARVO consensus committee could resolve such issues. Similarly, should a consensus be reached on standard culture conditions for trabecular or Schlemm’s canal cells? At present the comparisons of results from different laboratories can be difficult because of differences in culture media, serum supplements, and other culture conditions.

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Table 1

Understanding Aqueous Humor Outflow: Questions Needing Answers

Defining trabecular and Schlemm's canal cellular characteristics

1. Can specific cell markers be identified for trabecular and Schlemm's canal cells?
2. How do cells respond to environmental change (IOP, stretch, shear stress)?
3. What is the molecular composition of the tight junctions of Schlemm's canal cells? Does this differ from other endothella?
4. What is the effect of oxidative stress on trabecular and Schlemm's canal cells?
5. How do cells alter their formation and degradation of extracellular matrix in response to environmental changes? How rapidly?
6. What cellular dynamics lead to giant vacuole formation: role of the cytoskeleton and cell membrane? Is formation of giant vacuoles a general characteristic of all endothelial cells if perfused from basal to apical aspects of the cell?
7. Why are Schlemm's canal cells morphologically heteogeneic? Some cells form giant vacuoles, whereas others are flat. Is this a cellular phenomena or does it relate to aqueous flow pathways upstream?
8. What cellular dynamics lead to formation of the intercellular (B) and intracellular (I) pores in Schlemm's canal cells? Is their formation a property of the cell membrane when stressed or stretched?

Defining the role of the extracellular matrix in the outflow pathway

1. What is the role of individual extracellular matrix molecules in the aqueous outflow pathway? Can these be studied with gene-silencing techniques (i.e. antisense RNA, or siRNA)?
2. What is the phenotype of the aqueous outflow pathway in existing extracellular matrix protein knockout or knockin mouse models of extracellular matrix proteins?
3. Are advanced glycation end-products (AGE) present in the trabecular meshwork? What would be the effect on TM cells of an extracellular matrix that had AGE or oxidative modifications?
4. Do posttranslational protein modifications change in the trabecular meshwork with age and disease associations?
5. What causes the increase in outflow resistance with fixation in enucleated eyes? Is this due to changes in the extracellular matrix or to stiffening of the cells? Is this the same site that causes the increased outflow resistance in POAG?

Studying the uveoscleral pathway

1. What is the extent of outflow through the uveoscleral pathway when compared to total aqueous outflow in human eyes? Current values range from 4% to 60%. Does this percentage change with age? Stress?
2. How much aqueous is absorbed into choroidal blood? How much aqueous crosses the sclera and enters the orbit?
3. Can an imaging system be designed that would enable visualization of uveoscleral outflow, ideally in the living human?

Comparison of normal and POAG aqueous humor outflow

1. Are changes in aqueous humor proteins in POAG (i.e. TGFβ2, VEGF, endothelin, PAI, soluble CD44) associated with the disease or to the glaucoma medications that the individuals are taking?
2. Why does aqueous outflow resistance increase with age?
3. Is there a physiologic reason that POAG becomes increasingly common at about the same age that presbyopia develops?
4. Does aqueous outflow resistance change at night?
5. Do existing knock-out mice models have glaucoma-associated changes?

Models of outflow

1. Can animal models be designed and utilized in the study of the aqueous outflow pathway and POAG?
2. Are phenotype differences lost when cells are cultured in vitro?
3. Can cell cultures become more physiologic, matching cellular characteristics of fresh, noncultured cells? Identification of appropriate medium, substrate, and incubation conditions (i.e., low or room air oxygen levels) should be considered.